Environmental Health Perspectives
Vol. 49, pp. 135-140, 1983

Analysis at the Sequence Level of Mutations Induced by the Ultimate Carcinogen *N*-Acetoxy-*N*-2-Acetylaminofluorene

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The covalent binding of an ultimate carcinogen to the DNA bases or phosphate groups creates a premutational lesion that *in vivo* is processed by the repair, replication and recombination enzymes, and eventually may be converted into a mutation. Being interested in the way that an initial premutational event is converted into a stable heritable mutation, we have sequenced stable mutations in a gene that has formed covalent adducts *in vitro* with *N*-acetoxy-*N*-2-acetylaminofluorene (*N*-AcO-AAF, a model for the ultimate metabolite of the rat liver carcinogen 2-acetylaminofluorene, AAF). *In vivo* studies have shown the mutagenicity of AAF and its derivatives in both bacterial and eukaryotic systems. *N*-AcO-AAF reacts *in vitro* with DNA leading mainly to the formation of a guanine adduct, *N*-2-(deoxyguanosin-8-yl)-acetylaminofluorene (80%) and to at least three minor adducts. Studies by our group showed that binding of *N*-AcO-AAF to DNA resulted in a local distortion of the DNA helix around the C-8 adduct (the insertion-denaturation model).

We describe here the analysis of forward mutations induced in the tetracycline-resistance gene of pBR322 by directing the chemical reaction of the carcinogen to a small restriction fragment (*Bam*HI-*Sal*I) inside the antibiotic-resistance gene. Mutants are selected for ampicillin (Ap) resistance and tetracycline (Tc) sensitivity. The plasmid DNA of such mutants was analyzed for sequence changes in the fragment where the AAF binding had been directed.

We show here that the mutations are mainly frameshifts involving GC base pairs and that certain base pairs (hotspots) are affected at high frequencies.

Introduction

An important step in the carcinogenic process is thought to be the initial attack of the DNA molecule by a so-called ultimate carcinogen. In fact, more than 90% of the carcinogens tested are mutagens in bacterial systems (*1*). The premutational event is the covalent binding of the ultimate carcinogen to the DNA bases or phosphate groups. The chemical structure of the adducts formed, and to a lesser extent the structural changes induced in the DNA double helix in the neighborhood of the adducts, has been extensively studied during the last ten years. However the crucial question is "How will the different repair, replication and recombination enzymes handle these chemically modified bases?" In other words, since the end point of this initial step is a mutation, "How is this initial premutational event converted into a stable and heritable mutation?" *N*-Acetoxy-*N*-2-acetylaminofluorene (N-AcO-AAF) is a model ultimate metabolite of the strong rat liver carcinogen 2-acetylaminofluorene (AAF). *In vivo* studies have shown the mutagenicity of AAF and its derivatives in both bacterial (*2, 3*) and eukaryotic systems (*4*). *N*-AcO-AAF reacts *in vitro* with DNA leading mainly to the formation of a guanine adduct (*5*), *N*-2-(deoxyguanosin-8-yl)-acetylaminofluorene (80%) and also to at least three minor adducts (N. Schwartz, R. P. P. Fuchs and M. P. Daune, unpublished results), one of which is characterized as 3-(deoxyguanosin-N^2-yl)-acetylaminofluorene (*6*).

Studies from our group led to the general conclusion that binding of *N*-AcO-AAF to DNA resulted in a local distortion of the DNA helix around the C-8 adduct (*7-9*). We have called this structural alteration the insertion-denaturation model (*10*). A similar model has been proposed by other investigators (*11*).

In this paper we describe the analysis of forward mutations induced in the tetracycline-resistance

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either a single GC base pair or a doublet of adjacent GC-CG base pairs. Two of these mutants (numbers 35 and 36) also had a second mutation (Table 1).

Two hot spot sequences for mutagenesis were found within the collection of the nine mutants.

Hot Spot Sequence 1. As shown in Table 1, four out of the nine mutants (mutants number 4, 30, 41 and 45) show a deletion of a single G residue at position 520 or 521. (Numbering starts clockwise from the unique *Eco RI* site). It should be noted that the four mutants have arisen under quite different conditions (i.e., in two different strains, with or without ultraviolet induction of the SOS functions, with very different AAF modification levels).

Hot Spot Sequence 2. Mutants 34, as shown in Figure 3, and 36 exhibit a -2 deletion within the alternating GCGC sequence at positions 435-438 (deletion of a GC sequence at position 435-436 or 437-438, or of a CG sequence at position 436-437). Mutant 33 also exhibits a -2 deletion within a GCGC sequence at position 548-551. The mutation in mutant 35, although being different (-1 deletion of G 416 and double transition at 414-415) also takes place within the same 6-nucleotide long sequence, GGCGCC, sequence that is in common to all four mutants 34, 36, 33, 35. This given sequence, that is found three times within the 6S fragment, can therefore be considered as a mutational hot spot. Such a GC deletion in an alternating GC sequence was shown *in vivo* to be a hot spot for reversion of the mutation, his D 3052, in *Salmonella* by the mutagen 2-nitrosofluorene (*21*). It is striking to find that the same type of mutation occurs in both a reversion and a forward mutation assay.

Conclusion

The reason why such particular sequences are hot spots for mutagenesis is not clear. Whether or not such sequences are hot spots for the AAF binding reaction itself is presently under investigation. Alternatively, it is more likely that the processing of the premutational lesions is strongly sequence-dependent. It is noteworthy that at both hot spots the sequence is such that one can draw a short "hairpin-type" secondary structure (Fig. 4). Such hairpins are too short to be stable by itself but might be highly stabilized by the conformational change that -AAF introduces when bound to C-8 of guanine. As stated by the insertion-denaturation model proposed by Fuchs and co-workers (*7, 10*), there is a local denaturation of the helix around the

Table 1

Description of the mutation	Mutant number	Strain	Induction of SOS functions	Average number of bound AAF residues/6S fragment	Sequence in the neighborhood of the mutation[a]
- 1 deletion of G 520 or 521	4	AB 1886	+	2.0	511 520 529 G G G T A T G G T[G][G]C A G G C C C G - 1
	30	AB 1886	-	2.8	
	41	AB 1157	+	13.8	
	45	AB 1157	+	2.8	
- 1 deletion of G 389 or 390	32	AB 1157	+	8.8	381 390 399 T C T A C G C C[G][G]A C G C A T C G T - 1
- 2 deletion of GC 435-436 or 437-438 or CG 436-437	34	AB 1157	+	6.6	428 446 G T T G C T G[G][C][G][C]C T A T A T C G - 2
	36	AB 1157	+	8.8	
Addition of a C within sequence with 526-528	36				518 + 1 [C] 536 G T G G C A G G C C C G T G G C C G G
- 2 deletion of GC 548-549 or 550-551 or CG 549-550	33	AB 1157	+	13.8	540 558 A C T G T T G G[G][C][G][C]C A T C T C C - 2
- 1 deletion of of G 416 and double transition at 414-415	35	AB 1157	+	8.8	406 424 C A T C A C C G[G C][G]C C A C A G G T double ↑ -1 transition [A T]

[a]The sequences appearing in this table are the wild type sequences with the numbering defined by Sutcliffe (*20*). The bases involved in the deletion mutations are boxed with dotted lines. The different possibilities to obtain a given mutated sequence are shown. Mutant 36 exhibits two mutations: a - 2 deletion as in mutant 34 and a + 1 addition of a C residue within the sequence CCC at positions 526-528. Mutant 35 has got a - 1 deletion of a G at position 416 and a double transition, GC → AT, at position 414-415.

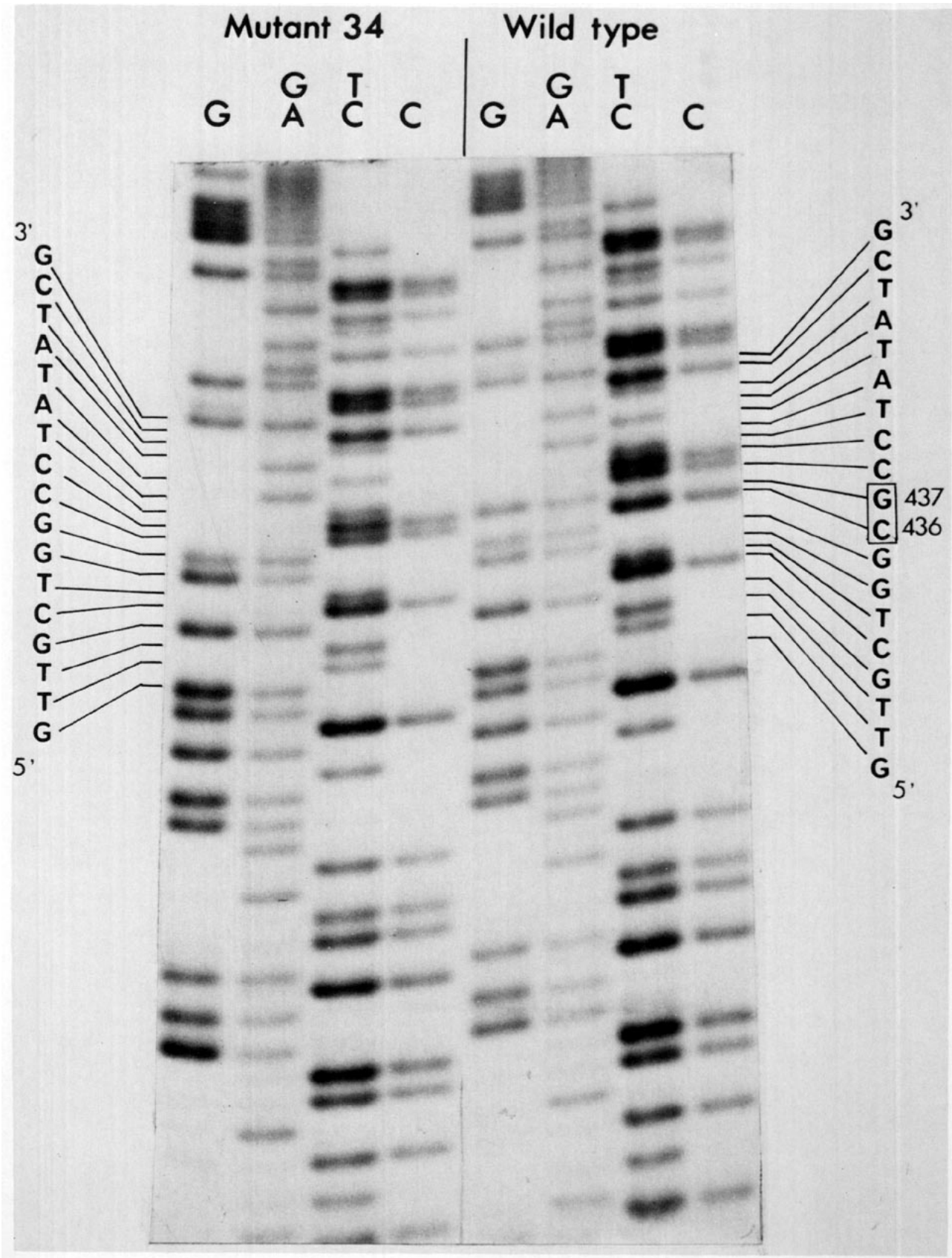

FIGURE 3. Part of the sequence of mutant 34 and of the wild type DNA showing the – 2 deletion of a CG doublet within the hot spot sequence GGCGCC.

guanine-AAF adduct that might favor the hairpin structure shown in Figure 4. Due to the multicopy state and to the recessivity of the mutations that are scored in our system, the conversion of the premutagenic lesion into a stable mutation most likely occurs simultaneously in both strands prior to replication.

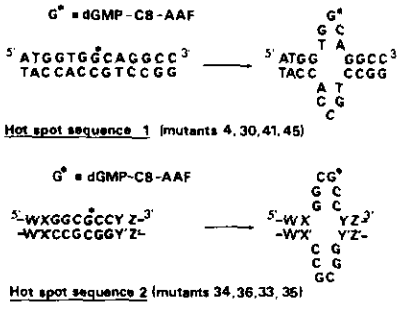

FIGURE 4. Hypothetical hairpin structure at hot spot sequences 1 and 2. According to the insertion-denaturation model proposed by Fuchs and co-workers (*7, 10*), there is a local denaturation of the helix around the guanine-AAF adduct that might favor the hairpin structure.

The molecular mechanism by which the mutation is being fixed remains to be elucidated.

This work has been supported by Grant No. 79.7.0664 from the D.G.R.S.T. (Délégation Générale a la Recherche Scientifique et Technique).

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